氏　　名	丁　　莉 莉
生 年 月 日	
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学位授与の題目	Characterization of HSP25,HSP43 and other small heat shock proteins in Caenorhabditis elegans (Caenorhabditis elegans（線虫）におけるHSP25,HSP43,およびその他の熱ショック蛋白質の性質）
論文審査委員(主査)	山口　和男（遺伝子実験施設・教授）
論文審査委員(副査)	福森　義宏（理学部・教授）中西　義信（薬学部・教授） 細野　隆次（医学部・教授）岩見　雅史（自然科学研究科・助教授）

学 位 論 文 要 旨

Abstract - Members of small heat shock proteins (sHSPs) in *C. elegans* are further characterized biochemically and immunohistochemically. HSP43, the largest member of the family, is expressed constitutively in all life cycle stages in *C. elegans* nematodes, prominently expressed in vulva, spermatheca, hypodermis or the structures of male tail. HSP25, the second largest member of the family, is expressed at all developmental stages under normal culture conditions, localized to the pharynx, spermathecal wall and body wall muscle. Recombinant HSP25 are small oligomers (dimer-tetramer), possesses chaperone activities in the standard assay, and binds to vinculin and α-actinin (not actin) by affinity chromatography. HSP16s, well-characterized proteins, are strictly stress-inducible, ubiquitously expressed throughout most somatic tissues in young animals, prominent in spermathecae, sperm and vulva in mature animals. HSP16s form large complex (24-mer). Mixed complex could be formed with two HSP16s in *E. coli*, and the mixed complex have same oligomer size and chaperone activities as their original counterparts. In another experiment, chimeric proteins were constructed from domains of different HSP16s, and the chimeric proteins also form large oligomers and possess chaperone activities. It was found that the N-terminal regions may be important for oligomerization and are buried inside the oligomer complex, while C-terminal extensions are exposed outside the complex. The binary assay suggests that the substrate-binding sites seem to be on the surface of the complex. HSP12s, the smallest members of the family, are expressed ubiquitously in young animals. Although overall levels are much lower in adult animals, HSP12s are locally abundant in specific vulval cells and sperm cells, likely to be in cytoplasmic localization of the sperm cells.

Introduction - Small heat shock proteins (sHSPs) are a group of proteins with low molecular weight, and sharing a conserved sequence called α-crystallin domain, and with variable N-terminal and C-terminal extensions. The subunits of sHSPs range from 12,000-43,000 of molecular weight, existing as large multimeric assemblies in solution (de Jong, *et al.*, (1998) *Int. J. Biol. Macromol.* 22, 151-162). The first three-dimensional structure of Hsp16.5 revealed a spherical array of 24 subunits of a polypeptide consisting largely of (-sheet (Kim *et*

al., (1998) *Nature* 394, 595-599). The chaperone activities of sHSPs could be studied upon their ability to prevent the aggregation or precipitation of denatured substrate proteins (Horwitz, J. (1992) *Proc. Natl. Acad. Sci. USA* 89, 10449-10453).

Some sHSPs are highly stress-inducible, and some are developmentally regulated, and some expressed constitutively. Studies revealed that mammalian sHSPs, hsp27, is overexpressed in the microfilament lattice against heat-induced disruptions, and actin might be a major target of the protective effect of hsp27, while this protective effect was dependent on the ability of hsp27 to be phosphorylated in vivo (Laszlo *et al.*, (1993) *Int. J. Radiat. Biol.* 63, 569-581; Lavoie *et al.*, (1993) *J. Biol. Chem.* 268, 3420-342; Lavoie *et al.*, (1993) *J. Biol. Chem.* 268, 24210-24214). Recently, hsp27 was found to interfere with apoptosis induced by tumor necrosis factor (Mehlen *et al.*, (1996) *EMBO J.* 15, 2695-2706), and this protective function was dependent on the formation of large hsp27 aggregates, although the formation of large oligomers are not phosphorylation-dependent (Mehlen *et al.*, (1997) *Biochem. Biophys. Res. Commun.* 241, 187-192; Suzuki *et al.*, (1998) *Plant Physiol.* 116, 1151-1161).

The completion of the C. elegans genome sequence (The C. elegans Sequencing Consortium (1998) Science 282, 2012-2018) provides a unique opportunity to investigate sHSP members in C. elegans. Using the protein sequence of HSP16-48 as query, a BLAST search of the C. elegans genome yields 16 genes encoding 14 distinct sHSPs, including HSP43, HSP25, HSP17.5 (not being characterized to date), SIP-1 (SEC-1), HSP16s (6 members), and HSP12s (4 members). Among them, HSP16s are strictly stress-inducible (Jones et al., (1986) J. Biol. Chem. 261, 12006-12015), and the others are constitutively expressed. HSP16s form large complex (24-mer), and are active in the standard chaperone assay. Deleting N-terminal of HSP16-2 results the size reducing of the complex and loss of chaperone activities, while HSP12s form dimer-tetramer and didn't show any chaperone activities in the standard assay (Leroux et al., (1997) J. Biol. Chem. 272, 24646-24656).

Results - To further understand sHSP family in C. elegans, HSP43 and HSP25 were characterized. Developmental profile experiments showed that HSP43 is expressed constitutively in all life cycle stages in C. elegans nematodes, prominently expressed in vulval region (utse and uv1 cells), spermathecal valve, and junctions between cells of the spermathecal cage. In body wall muscle of both N2 nematodes and him-8 mutants (high incidence of males), HSP43 forms a punctuated pattern of circumferential lines, likely corresponding to regions where the hypodermis contacts the muscle cells. In him-8 males, HSP43 is concentrated in specialized structures of the tail, including rays, copulatory spicules, hook and other structures. The complex size of HSP43 was estimated to be greater than 670 kDa, and forms multimers of at least 16 subunits.

HSP25 is expressed at all developmental stages under normal growth conditions. Recombinant HSP25 produced in E. coli exists predominantly as small oligomers (dimer-tetramer), and possesses chaperone activities against citrate synthase in the standard assay. In nematode C. elegans HSP25 is localized to dense bodies and M-lines in body wall muscle, to the lining of the pharynx, and to the junctions between cells of the spermathecal wall. The expression patterns were observed both at N2 nematodes and him-8 mutants. Affinity chromatography of nematode extracts on a column of immobilized HSP25 resulted in specific binding of vinculin and (-actinin but not actin, as revealed by Western blotting.

To further understand the functions of sHSPs, tissue specificity and developmental profiles of 10 sHSPs were examined with immunohistochemistry in N2 nematodes and him-8 mutants. The data showed that the sHSPs are prominently expressed in reproductive tissues in mature animals, including vulva (Hsp12s, HSP43, and HSP16s), spermathecae (HSP12s, HSP25,

HSP43, and HSP16s) and tail structures in male animals (HSP43). Under normal culture conditions, the tissue specific patterns were observed in HSP12s, HSP25 and HSP43, while the expression patterns of HSP16s could only be seen under heat shock.

The typical structure of sHSPs consists of three domains: variable N-terminal region, conserved (-crystallin, and variable C-terminal extension. To further understand the domain functions, the structure-function relationship was studied by constructing a series of chimeric proteins. The data showed that:

1. N-terminal region plays an important role in forming complex of subunits. When the C-terminal regions of HSP16s were replaced by the corresponding region of another HSP16, the chimeric proteins could form large oligomers and possess chaperone activities.
2. HSP16-2 and HSP16-41 form mixed complex when they were produced in the same cell in vivo, and the mixed complex has same oligomer size and chaperone activities as their original counterparts.
3. C-terminal extension is on the surface of the complex, since C-terminal His-tag hsp16s could bind to nickel resins. The data agree with the results of tryptic digestion when C-terminal extensions were cut by trypsin on native HSP16-2, N-terminal His-tag HSP16-2 and chimeric HSP16-48/2.
4. Complex form of HSP16-2 seems to be the chaperone active form.
5. The binding site of HSP16s seems to exist in α-crystallin domain. It is not excluded that C-terminal extension may be involved in the substrate-sHSP interaction. Present data further showed that the stoichiometry of substrate binding was 1:1 (monomer to monomer), which indirectly suggests that the substrates couldn't be bound inside.

Discussion - It was not surprised that HSP43 and HSP25 in C. elegans are related to muscle structures. Similar results are found in other organisms. Mammalian sHSPs are rich in muscle, and muscle is the tissue expressed all sHSPs studied so far (Sugiyama et al., (2000) J. Biol. Chem. 275, 1095-1104). Protein p27, a sHSP from the mammalian parasitic nematode Dirofilaria immitis, was found to be bound to the region immediately adjacent to the hypodermal membrane on the cytoplasmic side (Lillibridge et al., (1996) Exp. Parasitol. 83, 30-45). The data suggests that these proteins may perform closely related functions in different organisms, or as a general chaperone associated with muscle protein turnover, or in the maintenance of pre-formed structures within muscle cells. Other studies showed that the degradation of muscle proteins occurs via the ubiquitin-dependent proteasome pathway (Solomon et al., (1998) J. Biol. Chem. 273, 25216-25222), or via the ubiquitin-dependent system (Haas (1995) J. Biol. Chem. 270, 9407-9412).

Besides the expressions in muscles, most of the sHSPs studied are concentrated in reproductive tissues in mature animals (Wakayama and Iseki, (1999) Anat Embryol (Berl). 199, 419-425; Biggiogera et al., (1996) Exp. Cell. Res. 25, 77-85; Michaud et al., (1997) J. Cell. Sci. 110, 1989-1997). In the present data, all the sHSPs are more or less related to sperm/spermatheca, vulva or male tail structures. Our results demonstrate that a given C. elegans sHSP may be present in several different tissues, a given developmental stage, or different tissue specificity may vary at different stages. At this time it is not clear whether these distributions indicate that sHSPs perform different functions in different tissues, or whether they interact with a common or related set of target proteins.

It has been an intensive discussion topic that whether the size of the sHSPs complex is related to the chaperon activities. Present data showed that some sHSPs acquired chaperone activities as small oligomers (such as HSP25), and other sHSPs may loss or decrease

chaperone activities when the large oligomers are disrupted (such as HSP16-2), which suggests that N-terminal regions may be necessary for oligomer formation in some sHSPs. The structure-function studies suggest that the N-terminal regions are buried inside the complex, and the C-terminal extensions are exposed to the surface. Indirect data suggests that the binding sites are on the surface of the complex that may be related to α-crystallin domain. Our data also showed that mixed complex could be formed in experimental conditions between different sHSPs, but it's still not clear that whether sHSPs form mixed complex in the original cells, although it was found in mammalian cells (Sugiyama et al., (2000) J. Biol. Chem. 275, 1095-1104).

学位論文審査結果の要旨

本論文については，平成12年12月7日口頭発表を行い，終了後，審査委員会を開催し，以下の結論を得た。

本研究は全塩基配列が解読されている線虫(*C. elegans*)のゲノム中に，14種の異なる低分子熱ショック蛋白質(sHSPs)がコードされていることを見いだし，そのうちHSP43，HSP25，HSP16s，HSP12sについて，免疫組織化学的，生化学的，遺伝子工学的解析を行い次のような結果を得た。

HSP43は世代を通じて恒常的に合成されるが，特に陰門，貯精のう，真皮及び雄成虫の尾部の特殊な構造で発現していた。この蛋白質は分子量から少なくとも16量体で存在していた。HSP25も発生のどの時期でも合成されていたが，咽頭や貯精のう壁の細胞間などや，体を被う筋肉のデンスボディーとM線に局在していた。蛋白質間相互作用の実験から，HSP25はビンキュリンとα－アクチニンと結合するが，アクチンとは結合しないことが示された。更にシャペロンとしての活性も保持していた。また，HSP43，HSP25それぞれについて，RNAi法による遺伝子特異的な発現抑制をおこなったが，発生過程での異常は観察されなかった。

一方，HSP16sはストレスで合成の誘導が見られたが，全体の体細胞組織で発現し，老化と共に減少していった。これらの蛋白質は大きな複合体（24量体）を形成し，シャペロン活性も検出された。HSP16-2ではN末端側に多量体形成能やシャペロン活性が担われていた。また，これらの活性を保持したままで，異なるHSP16蛋白質同志でドメインを交換したキメラ蛋白質を作成することができた。

HSP12sは若い成虫で恒常的に作られ，特に陰門や精子の細胞質に存在していた。この蛋白質は2-4量体を形成していた。しかし，シャペロン活性は見いだされなかった。

以上のように，本研究は多面的な解析により，低分子熱ショック蛋白質について，線虫ばかりでなく，より一般的なこれら蛋白質の構造と機能を考える上で示唆に富む結果を示しており，充分博士論文に値すると判定した。